Evaluation of endometrial biomarkers for semi-invasive diagnosis of endometriosis

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Objective: To test the hypothesis that specific proteins and peptides are expressed differentially in eutopic endometrium of women with and without endometriosis and at specific stages of the disease (minimal, mild, moderate, or severe) during the secretory phase.
Design: Patients with endometriosis were compared with controls.
Setting: University hospital.
Patient(s): A total of 29 patients during the secretory phase were selected for this study on the basis of cycle phase and presence or absence of endometriosis.
Intervention(s): Endometriosis was confirmed laparoscopically and histologically in 19 patients with endometriosis of revised American Society for Reproductive Medicine stages (9 minimal-mild and 10 moderate-severe), and the presence of a normal pelvis was documented by laparoscopy in 10 controls.
Main Outcome Measure(s): Protein expression of endometrium was evaluated with use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. The differential expression of protein mass peaks was analyzed with use of support vector machine algorithms and logistic regression models.
Result(s): Data preprocessing resulted in differential expression of 73, 30, and 131 mass peaks between controls and patients with endometriosis (all stages), with minimal-mild endometriosis, and with moderate-severe endometriosis, respectively. Endometriosis was diagnosed with high sensitivity (89.5%) and specificity (90%) with use of five down-regulated mass peaks (1.949 kDa, 5.183 kDa, 8.650 kDa, 8.659 kDa, and 13.910 kDa) obtained after support vector machine ranking and logistic regression classification. With use of a similar analysis, minimal-mild endometriosis was diagnosed with four mass peaks (two up-regulated: 35.956 kDa and 90.675 kDa and two down-regulated: 1.924 kDa and 2.504 kDa) with maximal sensitivity (100%) and specificity (100%). The 90.675-kDa and 35.956-kDa mass peaks were identified as T-plastin and annexin V, respectively.
Conclusion(s): Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis of secretory phase endometrium combined with bioinformatics puts forward a prospective panel of potential biomarkers with sensitivity of 100% and specificity of 100% for the diagnosis of minimal to mild endometriosis. (Fertil Steril® 2011;95:1338–43. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometrium, proteomics, SELDI-TOF-MS, secretory phase, endometriosis, support vector machine

Endometriosis, defined as the ectopic presence of endometrial-like tissue, is an enigmatic, benign, estrogen-dependent disease, associated with infertility and pain. It is progressive in 40% to 50% of women (1), and recurrence of endometriosis is often observed after surgery and after cessation of medical therapy, especially in women with moderate to severe endometriosis (2). Endometriosis is associated with a high cost (2–4), estimated to be higher than the cost for Crohn's disease (4).

Early detection of endometriosis is crucial for its timely diagnosis and treatment. Studies report an average delay of 11.7 years in the United States and 8.0 years in the United Kingdom (5) before women get a correct diagnosis after the initial onset of symptoms for endometriosis. However, endometriosis can be diagnosed only via laparoscopy, ideally combined with histologic confirmation. A noninvasive or semi-invasive diagnostic test in easily accessible fluid or tissue (i.e., plasma, serum, urine, saliva, endometrium) would be beneficial to both physicians and patients but does not exist (6–9).

Protein analysis using two-dimensional gel electrophoresis or more advanced technology may represent a promising method for developing noninvasive diagnosis of endometriosis, on the basis of

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previous reports showing differential protein expression in women with endometriosis when compared with controls in peritoneal fluid (10, 11) or in endometrium (12–15). Although two-dimensional electrophoresis has high-resolution capacity, it is labor intensive and requires large quantities of intact proteins. Protein profiling using surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) allows study of the expression of peptides and proteins that are poorly detected by other analytic methods, but precautions should be taken when designing SELDI-TOF-MS experiments to avoid bias in data interpretation (16). Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry was used initially (17) to identify biomarkers for ovarian cancer. Although this first study had some serious flaws (18), the SELDI-TOF-MS technique has been improved over the years and has been used to identify biomarkers for women with mutations of breast cancer 1, breast cancer (19), and early ovarian cancer (20). In our preliminary study, SELDI-TOF-MS protein profiling of eutopic endometrium showed that several proteins and peptides were expressed differentially in women with endometriosis when compared with controls (21), suggesting that proteomic analysis of endometrium may be a promising method for the diagnosis of endometriosis. The aim of this study was to test the hypothesis that specific proteins and peptides are differentially expressed by eutopic endometrium of women with and without endometriosis and at specific stages of the disease (minimal, mild, moderate, or severe) during the secretory phase.

MATERIALS AND METHODS

Patients and Tissue Selection

The biobank of the Leuven University Fertility Center was searched to identify 10 endometrial samples obtained during the secretory phase (day 16–26 of a 28-day menstrual cycle (22) from each of the following three groups: women with a normal pelvis (controls, n = 10), women with minimal to mild endometriosis (stages I–II, n = 9), and women with moderate to severe endometriosis (stages III–IV, n = 10). Endometriosis was staged according to the classification system of the American Society for Reproductive Medicine (23) during laparoscopy and confirmed by histopathology. Endometrial samples from patients with and without endometriosis had been collected retrospectively between April 2003 and July 2005 by endometrial biopsy during hysteroscopy or laparoscopy procedures for subfertility with or without pain (Table 1) and had been frozen at −80°C until use. All patients were white, with similar age among women with endometriosis (mean 31.7 ± 4.2 years, median 30 years, range 27–40 years) and controls (mean 31.5 ± 6.0 years, median 31.5 years, range 23–41 years). All patients had signed a written informed consent before surgery and had agreed on the collection of tissues for research. The study protocol had been approved by the institutional ethical and review board of the University Hospital Gasthuisberg for the protection of human subjects.

Patients with pelvic inflammatory disease, myomas, or urinary tract infection; patients using the oral contraceptive pill; patients taking long-term medication; and patients operated on within 6 months before the time of sample collection were excluded from this study. Only endometrial samples obtained during the secretory phase were selected for this study to rule out cycle-dependent changes in endometrial protein or peptide expression. One control patient was noted to have subacute focal endometritis. A blind screening approach was applied on these samples with use of SELDI-TOF-MS to search for potential biomarkers.

Preparation of Protein Lysate From Endometrial Samples

Homogenization of tissue Frozen endometrial tissue biopsy samples were weighed (100 mg/mL lysis buffer) and immediately thawed in phosphate-buffered saline solution while on ice. Tissues were washed five times in phosphate-buffered saline solution to rinse off any adhering hemoglobin. The tissue homogenization was realized as previously described (21), followed by hemoglobin depletion (Supplemental Materials and Methods).

Statistical analysis: data preprocessing The SELDI-TOF mass spectra were baseline corrected and normalized on the basis of total ion current with use of the Biomarker Wizard Program (Ciphergen, Fremont, CA). The same application was used for peak detection and the determination of *P* values. For *P* value calculations, peaks exceeding a peak threshold of 20% of the total ion current and exhibiting a signal-to-noise ratio of at least 3 were selected and analyzed with the nonparametric Mann-Whitney *U* test. All univariate analyses were carried out with use of ProteinChip Software (v3.1.1; Ciphergen) and the Prism 3 software (GraphPad, San Diego, CA). Results are expressed as mean ± SD, median, range. A differentially expressed mass peak with P value $<.05$ was considered to be statistically significant. Multivariate analysis was applied to evaluate and identify potential biomarkers with diagnostic value. Feature selection was accomplished through a support vector machine (SVM)–based feature ranking algorithm (24), stepwise logistic regression, and logistic ridge regression (25) to rank the selected mass peaks according to their classification power. The performance of these models was evaluated with use of leave-one-out cross validation (LOO-CV) to avoid overfitting. Subsequently, the highly ranked mass peaks overlapping between these three models (selected via simple stepwise logistic regression, with an odds ratio of at least 2 in logistic ridge regression, and highly ranked by the SVM were selected and their performance was checked by a logistic ridge regression model with 10-fold CV.

RESULTS

Mass Peak Expression in Women With Endometriosis Compared With Controls

After preprocessing the mass spectra with use of the Ciphergen ProteinChip Software, 73 mass peaks were expressed differentially in the secretory endometrium of all women with endometriosis (stages I–IV) compared with all controls. The mass of the differentially expressed peaks ranged between 1.923 kDa and 133.810 kDa. Peaks with a mass-over-charge <1.6 kDa were excluded to avoid interference from matrix ions. To select a set of candidate biomarkers, two models based on logistic regression were built. The first model (LOO–stepwise logistic regression model) selected 14 peaks with a performance of 76% (LOO-CV), and the second model (LOO–logistic ridge regression odds ratio >2) selected 16 peaks

TABLE 1

Demographic data of women with and without endometriosis with indications for laparoscopic surgery.

Subjects	Total no. of subjects	Infertility symptoms		Pain symptoms	
		Yes, n (%)	No, n (%)	Yes, n (%)	No, n (%)
Controls	10	9 (90)	1 (10)	3 (30)	7 (70)
Endometriosis stage I–II	9	7 (77.8)	2 (22.2)	7 (77.8)	2 (22.2)
Endometriosis stage III–IV	10	10 (100)	0	7 (70)	3 (30)

Kyama. Biomarkers for early detection of endometriosis. Fertil Steril 2011.